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Infectious cDNA Clone of Attenuated Langat Tick-Borne Flavivirus (Strain E5) and a 3′ Deletion Mutant Constructed from It Exhibit Decreased Neuroinvasiveness in Immunodeficient Mice

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Forty-five years ago a naturally attenuated tick-borne flavivirus, Langat (LGT) strain TP21, was recovered from ticks in Malaysia. Subsequently, it was tested as a live attenuated vaccine for virulent tick-borne encephalitis viruses. In a large clinical trial its attenuation was confirmed but there was evidence of a low level of residual virulence. Thirty-five years ago further attenuation of LGT TP21 was achieved by multiple passages in eggs to yield mutant E5. To study the genetic determinants of the further attenuation exhibited by E5 and to allow us to manipulate the genome of this virus for the purpose of developing a satisfactory live attenuated tick-borne flavivirus vaccine, we recovered infectious E5 virus from a full-length cDNA clone. The recombinant E5 virus (clone 651) recovered from a full-length infectious cDNA clone was more attenuated in immunodeficient mice than that of its biologically derived E5 parent. Increase in attenuation was associated with three amino acid substitutions, two located in the structural protein E and one in nonstructural protein NS4B. Subsequently an even greater degree of attenuation was achieved by creating a viable 320 nucleotide deletion in the 3′-noncoding region of infectious full-length E5 cDNA. This deletion mutant was not cytopathic in simian *Vero* cells and it replicated to lower titer than its E5-651 parent. In addition, the E5 3′ deletion mutant was less neuroinvasive in SCID mice than its E5-651 parent. Significantly, the deletion mutant proved to be 119,750 times less neuroinvasive in SCID mice than its progenitor, LGT strain TP21. Despite its high level of attenuation, the E5 3′ deletion mutant remained highly immunogenic and intraperitoneal (ip) inoculation of 10 PFU induced complete protection in Swiss mice against subsequent challenge with 2000 ip LD50 of the wild-type LGT TP21.

INTRODUCTION

Langat virus (LGT) belongs to the tick-borne encephalitis virus (TBEV) complex, within the genus *Flavivirus* of the family *Flaviviridae*. There are more than 60 antigenically related, positive-strand RNA flaviviruses, many of which cause considerable morbidity and mortality in humans (Calisher *et al.,* 1989; Monath and Heinz, 1996). Langat virus initially recovered from ticks in Malaysia did not appear to be associated with human disease under natural conditions (Smith, 1956). Also, LGT has been shown to be effective in inducing cross-reactive neutralizing antibodies and in preventing disease caused by tick-borne encephalitis complex flaviviruses in experimental animals (Nathanson *et al.,* 1968; Price *et al.,* 1970; Price and Thind, 1973; Pletnev *et al.,* 2000). For this reason, LGT virus was considered as a live attenuated vaccine for prevention of tick-borne encephalitis which is a widespread disease in many European and Asian countries. Several LGT derivatives were isolated and shown to be partially attenuated for mice and monkeys (Thind and Price, 1966a; Il'enko *et al.,* 1968; Mayer *et al.,* 1976; Smoridincev and Dubov, 1986). One such strain, designated E5, was selected by 42 passages of the wild LGT TP21 strain in embryonated chicken eggs (Thind and Price, 1966a,b). LGT E5 virus differed from the wild LGT TP21 strain by its lower peripheral neurovirulence ("neuroinvasiveness") for mice, low neurovirulence in *Ateles geoffroyi* monkeys inoculated intracerebrally, as well as *Macaca mulatta* monkeys inoculated intraspinally. Based on assessment of histological lesions in central nervous system (CNS) of rhesus monkeys, the attenuated LGT E5 strain exhibited reduced neurovirulence that was less than that of 17D yellow fever virus vaccine but greater than that of the attenuated poliovirus type 3 oral vaccine strain (Nathanson *et al.,* 1968).

Recently, the complete sequence of wild-type LGT virus (TP21 strain) genome and its more attenuated E5 derivative was determined (Campbell and Pletnev, 2000). There were 12 nucleotide differences in the consensus sequence of the two LGT strains (parental TP21 and its derivative E5), of which seven produced an amino acid substitution in the envelope structural protein E ($Phe_{119} \rightarrow Val$ and $Asn_{389} \rightarrow Asp$), nonstructural protein NS3 ($Asn_{22} \rightarrow Ser$, $Phe_{248} \rightarrow Tyr$ and $Phe_{317} \rightarrow Leu$), and NS5 ($Ser_{422} \rightarrow Thr$ and $Arg_{542} \rightarrow Lys$). These mutations

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were associated with a difference in neuroinvasiveness of these LGT strains in Swiss mice observed earlier (Pletnev and Men, 1998). LGT TP21 was at least 2000 times more neuroinvasive than its E5 derivative when tested in immunocompetent mice. Only 10–20% of adult Swiss mice inoculated intraperitoneally with 10^7 PFU of E5 developed signs of encephalitis. Both strains were highly immunogenic in animals. A low dose of either TP21 or E5 (i.e., 10 or 10^2 PFU) was immunogenic as measured by induction of serum neutralizing antibodies (Pletnev and Men, 1998). Significantly, infection with LGT TP21 or E5 provided protection against challenge with tick-borne encephalitis virus (European or Far Eastern strain; Pletnev *et al.,* 2000). Nevertheless, LGT E5 exhibits very little evidence of neuroinvasiveness in normal mice even when inoculated in high dose. Ideally, live attenuated tick-borne flavivirus vaccines should be free of this very low level of residual virulence. Thus, before considering the attenuated E5 strain of LGT as a possible candidate for use in prophylaxis of human TBE disease, we attempted to remove the last vestiges of peripheral neurovirulence of LGT E5 for experimental animals by constructing a full-length infectious clone of LGT E5. The availability of such an infectious clone would allow us to introduce site-specific mutations into the infectious cDNA clone and evaluate their effect on virulence.

Previously, we found that the immunodeficient mouse to be an extraordinarily sensitive assay system for detecting low levels of residual virulence of LGT E5. The ability of SCID mice to detect neuroinvasiveness of E5 was approximately 10^8 greater than was the case for normal mice. In the present study an infectious cDNA clone of LGT E5 was constructed and used to identify mutations that diminished neuroinvasiveness of the already partially attenuated LGT E5 mutant without demonstrable diminution of immunogenicity or protective efficacy.

RESULTS

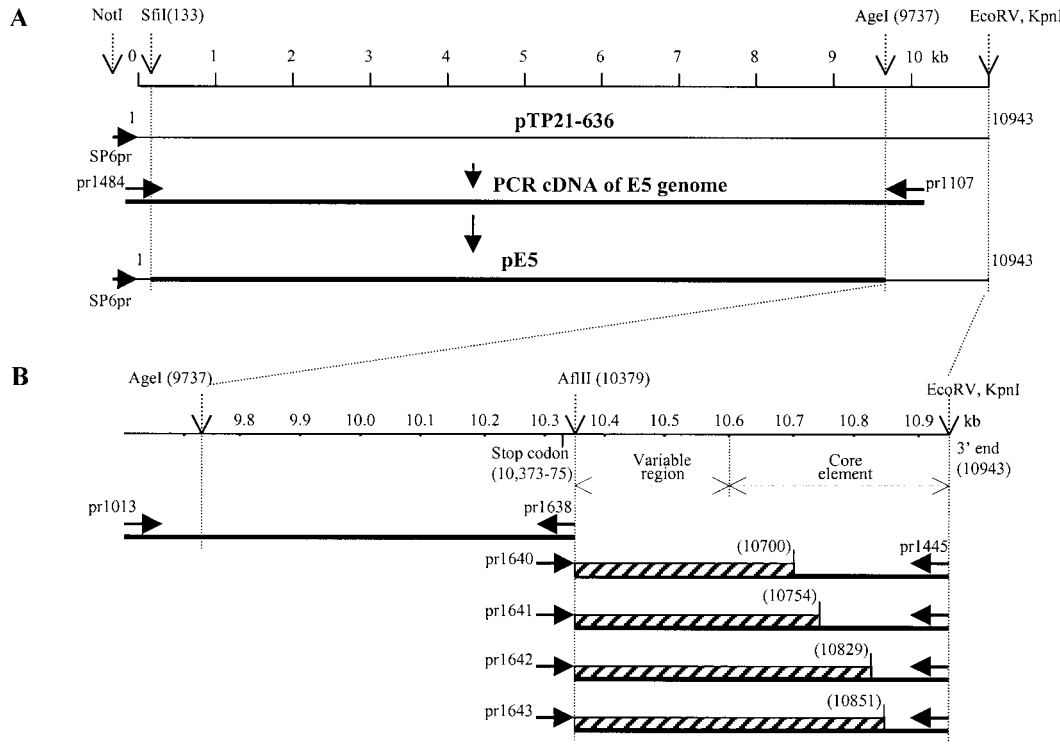
Construction of full-length E5 cDNA clone and recovery of virus from cells transfected with full-length RNA transcripts

Recently we succeeded in constructing stable LGT TP21 full-length cDNA clones from which infectious RNA could be transcribed *in vitro* (Campbell and Pletnev, 2000). One of these full-length cDNA clones, plasmid pTP21–636, was used to create full-length cDNA of E5 genome by replacing almost the entire TP21 genome with the corresponding sequence of E5 strain. The complete nucleotide sequence of the wild-type LGT virus (TP21 strain) genome and its more attenuated derivative, strain E5, was determined previously from cDNA fragments produced by RT-PCR (GenBank accession nos. AF253419 and AF253420). The TP21 and E5 genomes were both 10,943 nucleotides (nt) in length and contained a 130 nt 5′ noncoding region (NCR) and a 568 nt 3′ NCR that were completely conserved. Twelve differences in genome sequence of TP21 and E5 strain were located between nucleotide positions 1325 and 9288. This region together with flanking conserved sequences (nts 133 to 1324 and nts 9289 to 9737) in the infectious cDNA of cloned full-length TP21 genome was targeted for substitution with the corresponding sequence of E5 (Fig. 1A). An almost full-length cDNA fragment (~10.5 kb) of the E5 genome was prepared by high-fidelity long PCR using an RT product derived from viral RNA extracted from a low-titered E5 virus stock (1.2×10^4 PFU/ml). Low-titered virus harvested early in the growth cycle was used to prepare cDNA as template for PCR to minimize the presence of mutants with large 3′ deletions or rearranged genomes that accumulate late in infection as observed previously (Campbell and Pletnev, 2000). Conservation of the 5′ 132 nts and the 3′ 1205 nts of TP21 and E5 allowed us to clone the PCR product of the E5 genome, spanning nts 133 to 9737, into the pTP21–636 vector replacing the corresponding TP21 sequence. Six stable full-length E5 cDNA clones were identified by restriction enzyme digestion pattern. The partial sequence of these plasmids (pE5) was analyzed and found to contain the E5-specific sequences that differentiate E5 from its TP21 parent (Campbell and Pletnev, 2000).

Prior to producing run-off transcripts, the plasmid DNA template was linearized using *Eco*RV, whose cleavage site is present three nucleotides downstream of the 3′ end of LGT E5 sequence. Full-length RNA generated by SP6 polymerase from six different plasmids was tested for infectivity by transfection of CEF cells or simian *Vero* cells. Only one E5 cDNA clone (pE5-651) was infectious for both cell lines while the other clones were not viable. Evidence of virus infection was detected by IFA with LGT-specific hyperimmune mouse ascitic fluid (HMAF). All of the transfected *Vero* cells and 20–30% of CEF-transfected cells were positive on day 5. Stock preparations of the rescued E5 clone were produced by passaging the virus once or 12 times in the cell line used for rescue and harvesting the supernatant medium of infected cultures. After one or 12 passages in *Vero* or CEF cells, virus was analyzed for deviation of sequence from its biologically derived E5 parent.

Genetic variability of E5-651 virus during construction, recovery, and passage in cell culture

The complete sequence of the E5-651 virus genome rescued from cDNA in *Vero* or CEF cells was determined by analysis of overlapping RT-PCR cDNA fragments, derived directly from virus RNA, and compared with the consensus sequence of its parental E5 virus (Campbell and Pletnev, 2000) as well as the nucleotide sequence of the viral insert in the pE5-651 plasmid from which infectious RNA transcripts were derived (Table 1). The res-



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Size of deletion (nts)	Sequence of junction	Intermediate plasmid construct	Full-length E5 cDNA clone
320	10,379 10,700 5'-CAATATTTTAAAGCCTTAAGAAACTTTGTG-3'	p624-3'-320	pE5-3'-320
374	10,379 10,754 5'-CAATATTTTAAAGCCTTAAGGCCCCCAGGG-3'	p624-3'-374	pE5-3'-374
449	10,379 10,829 5'-CAATATTTTAAAGCCTTAAGATACCAAATTC-3'	p624-3'-449	pE5-3'-449
471	10,379 10,851 5'-CAATATTTTAAAGCCTTAAGGGGGGGCGGT-3'	p624-3'-471	pE5-3'-471

FIG. 1. Construction of full-length cDNA of LGT E5 genome. (A) The assembly of full-length cDNA of E5 in a plasmid was performed using the pTP21-636 which was cloned and sequenced as described earlier (Campbell and Pletnev, 2000) and the *Sfi*I(133)–*Age*I(9737) fragment that was derived by long PCR. (B) Construction and location of the deletions in the 3′-NCR of E5 genome. Position of the cleavage site of *Not*I, *Sfi*I, *Age*I, *Afl*II, *Kpn*I, and *Eco*RV in the cDNA shown in (A) or (B) by dashed lines. Solid lines indicate PCR cDNA fragments derived from the E5 genome. Short horizontal arrows indicate position of SP6 promoter or position of primer; vertical solid arrows indicate subsequent steps in cloning strategy. The numbers at the ends of LGT cDNA fragments represent the first and the last nucleotide positions of the genome, respectively. NT numbering derived from the results of RT-PCR sequence of E5 genome (GenBank accession no. AF253420). The position of the introduced deletion into the 3′-NCR, which extends from nt 10379 to the position indicated on top of the striped boxes, is shown. (C) Sequence of the 3′-NCR junctions of the deletion mutant cDNA genomes. The LGT E5 nucleotide sequence is in bold letters. The *Afl*II cleavage site, which was used to generate deletions, and TAA-stop codon indicated by the underlined sequence. The size of deletion, its position, and corresponding plasmid construct is shown.

cued E5-651 clone contained the E5 consensus sequence in the 12 positions at which the wild-type TP21 parent differed from its E5 derivative. Analysis of the plasmid DNA revealed eight differences in nucleotide sequence from the consensus sequence of E5, of which three produced an amino acid substitution in the envelope structural protein E ($Glu_{149} \rightarrow Gly$ and $Glu_{291} \rightarrow Gly$) and nonstructural protein NS4B ($Ala_{183} \rightarrow Val$).

The sequence of the first passage *Vero* cell grown clone E5-651 did not differ from the plasmid cDNA sequence, whereas after 12 passages in *Vero* cells fluctuation between (i) Ser_{17} and Asn and (ii) Gln_{383} and Lys was identified in NS2B and NS3, respectively. E5-651 rescued and passaged once in CEF cells differed from the plasmid DNA sequence at 2 nt positions in E; one of the changes resulted in fluctuation between C and U at

TABLE 1

Changes from Consensus Sequence of E5 That Occurred during Cloning, Rescue of E5 from Full-Length cDNA, and Passage in Simian *Vero* or Chicken Embryonic Fibroblast (CEF) Cell Culture

Genome region	NT position	Sequence of parental E5	Plasmid pDNA	Deviation from E5 consensus sequence: Rescued E5-651 isolated or passaged in *Vero* Passage 1	*Vero* Passage 12	CEF Passage 1	CEF Passage 12
5′NCR	53	U					C
E	1151	C				C/U	U
	1358	C					U (His_{130} → Tyr)
	1415	G				G/A (Glu_{149}/Arg)	A (Glu_{149} → Arg)
	1416	A	G (Glu_{149} → Gly)	→	→	→	→
	1485	C					C/U(Thr_{172}/Ile)
	1842	A	G (Glu_{291} → Gly)	→	→	→	→
NS1	3046	U	C	→	→	→	→
	3154	U	C	→	→	→	→
NS2B	4254	G			G/A (Ser_{17}/Asn)		
NS3	4528	U	C	→	→	→	→
	4675	C					A
	5744	C			C/A (Gln_{383}/Lys)		
NS4B	6955	C	U	→	→	→	→
	7294	G	A	→	→	→	→
	7455	C	U (Ala_{183} → Val)	→	→	→	→

Note. Coding changes are positioned left of center whereas non-coding changes are positioned in the center of their respective columns. Long arrow denotes a coding or non-coding change that is conserved subsequently.

nt position 1151 of E and fluctuation between Gly_{149} and Arg at nt position 1415 of E. When CEF cell culture-derived virus was passaged an additional 11 times in CEF cells, only U (nt 1151) or A (nt 1415) was selected from the fluctuation mixture. In addition, four other substitutions were identified, two of which a produced a coding change (Table 1). In contrast, only two positions 4254 (G/A) and 5744 (C/A) in genome sequence varied after 12 passages of E5-651 virus in *Vero* cells. Because the frequency of E5-651 genomic changes in CEF cells was greater than that observed in *Vero* cells, the *Vero* cell culture-derived E5-651 virus was selected on the basis of its apparent greater stability to be used for analysis of mouse neuroinvasiveness. Before evaluating virus virulence in mice, E5-651 virus was subjected to plaque-to-plaque purification to minimize the accumulation of spontaneous mutations, which might occur during virus amplification in *Vero* cells.

Plaquing efficiency and purification of E5 clone in cell culture

Plaque phenotype of the rescued E5-651 virus recovered from *Vero* cells and passaged once in these cells was examined using simian $LLCMK_2$ and *Vero* cells. The rescued *Vero* cell culture-derived E5-651 virus produced small clear transparent plaques 1.5 mm in diameter on $LLCMK_2$ cells 7 days postinfection (Table 2). In contrast, this virus produced smaller (<0.1 mm) faint plaques in *Vero* cells. In comparison, parental E5 virus grown in *Vero* cells produced large plaques (5.0–5.2 mm) on both *Vero* and $LLCMK_2$ cells. Clone E5-651 rescued and grown in CEF cells exhibited the same plaque size and morphology in the two simian cell cultures as *Vero* cell-derived virus (data not shown).

Individual 1.5-mm plaques of E5-651 were harvested from $LLCMK_2$ cells infected with *Vero* cell culture-derived virus and then subjected to three additional plaque-to-plaque passages in $LLCMK_2$ cells. Seed stock of the plaque-purified isolate of E5-651 virus was prepared following further amplification in *Vero* cells. Difference of plaque phenotype of the E5-651 virus in *Vero* and $LLCMK_2$ cells did not change following plaque-to-plaque selection in $LLCMK_2$ cells and amplification in *Vero* cell culture. In addition, the plaque-purified isolate did not differ in sequence from its rescued E5-651 virus.

Evaluation of cDNA-derived E5 virus in mice

Previously, wild-type strain TP21 was shown to be virulent for 3-week-old Swiss mice with an intraperitoneal LD50 of 5×10^3 PFU (Pletnev and Men, 1998). In contrast, the attenuated E5 strain derivative of TP21 exhibited neuroinvasiveness in adult mice only when the amount of virus inoculated was increased to 10^7-10^8 PFU. In the earlier study SCID mice were shown to be at least

TABLE 2

Lineage and Reduction of Neuroinvasiveness of Langat Virus (LGT) during Passage in Eggs and Subsequent Recovery from Full-Length cDNA and Deletion of 320 nt from Its 3′ Non-Coding Region

LGT strain	Derivation	Plaque size[a] (mm) in simian cells		Titer (FFU/ml)[b] attained in cell culture at 5 days		Neuroinvasiveness of *Vero* cell-grown virus for SCID mice inoculated ip		
							Fold reduction compared to	
		$LLCMK_2$	*Vero*	$LLCMK_2$	*Vero*	LD_{50} (PFU)	Immediate parent	TP21
TP21	Isolated from ticks	5.0	4.0	2.8×10^8	1.9×10^9	0.004[c]		
E5	42 passages in eggs	5.0	5.2	2.4×10^7	2.3×10^8	0.06[c]	15	15
E5-651	Recovered from full-length cDNA in *Vero* cells	1.5	<0.1	4.4×10^6	1.7×10^8	20.4	340	5,100
E5-3′-320	320 nt deletion in 3′ NCR of genome recovered in *Vero* cells	~0.2	No plaques	5.0×10^5	1.5×10^4	479	23.5	119,750

[a] Plaque size on day 7 post-infection.

[b] Data were taken from growth curve analysis (shown in Fig. 2) where MOI was 0.01 and virus titer in medium of infected cells was determined by a day 5 immunostaining focus-forming assay (see Materials and Methods).

[c] Data from Pletnev and Men, 1998.

10^6-10^8 times more permissive than normal mice for detection of peripheral neurovirulence of Langat virus strains. For this reason, three-week-old SCID mice in groups of 5 were inoculated ip with decimal dilutions of E5-651 or with 1 PFU of parental E5 strain whose LD50 had previously been determined to be 0.06 PFU (Pletnev and Men, 1998). 10^5 PFU of chimera TP21/DEN4(vac) was also evaluated ip in SCID mice as a negative control because a previous study indicated that it lacked any evidence for neuroinvasiveness (ip LD50 of $>10^7$ PFU; Pletnev and Men, 1998; Pletnev *et al.,* 2000). Parental E5 virus caused 100% mortality within 9 to 13 days after ip inoculation of 1 PFU, whereas 10^5 PFU of the chimeric virus failed to cause fatal disease over a period of 7 weeks. However, 2 of 5 mice died 28 or 33 days following ip inoculation of 100 PFU of E5-651. In a subsequent experiment, groups of five SCID mice were inoculated ip with decimal dilutions of clone E5-651, and its LD50 was determined to be 20.4 PFU (21.0 PFU in repeat experiment). Thus, clone E5-651 was 5.1×10^3 times less neuroinvasive than strain TP21 which had an estimated LD50 for SCID mice of 0.004 PFU and 3.4×10^2 times less virulent than its immediate parental E5 virus which had an estimated LD50 of 0.06 PFU for SCID mice (Pletnev and Men, 1998).

Construction of 3′-NCR deletion mutants and virus recovery

In an attempt to increase the level of attenuation of clone E5-651 for SCID mice, several strategic mutations were introduced in its cDNA genome. With regard to the development of an attenuated live flavivirus vaccine, any attenuating mutations introduced into the candidate vaccine virus should be genetically stable and not able to effect a significant reduction in immunogenicity and protective efficacy. Recent studies involving several flaviviruses suggest that 3′-NCR deletions meet these requirements (Men *et al.,* 1996; Mandl *et al.,* 1998). The 3′-NCR of the RNA genome of tick-borne flaviviruses varies from 393 to 800 nt in length, of which only the last approximately 340 nts (core element) are more conserved than the region between the stop codon of the open reading frame and the core element (Dobrikova and Pletnev, 1995; Mandl *et al.,* 1998). A recent study of TBEV provided evidence that a 3′-NCR deletion could reduce virulence without loss of viability if the deletion extended from the stop codon of the viral polyprotein to the beginning of the core element (Mandl *et al.,* 1998).

We introduced deletions that start at the fifth nucleotide following the TAA-stop codon of the long open reading frame and extend to targeted nucleotides indicated in Figs. 1B and 1C. All mutant constructs contained an additional three or five nucleotides that created an *Afl*II restriction enzyme cleavage site at the site of deletion (Fig. 1C). The final mutant plasmids pE5-3′-320, pE5-3′-374, pE5-3′-449, and pE5-3′-471 contained a deletion 320, 374, 449, or 471 nt in length, respectively.

Vero cells were transfected with full-length genomic RNA transcripts prepared from full-length cDNA of the 3′-NCR deletion mutants described above. Only E5-3′-320 mutant RNAs yielded viable virus. These results were consistent with those observed for TBEV 3′-NCR deletion mutants (Mandl *et al.,* 1998). Extension of deletion into the core element of the 3′ end TBEV or LGT genome abolishes virus viability. The longest deletion of TBEV compatible with viability retains the last 222 nts of the 3′ end. The 3′-NCR of rescued E5-3′-320 virus contains the last 244 nts of its genome.

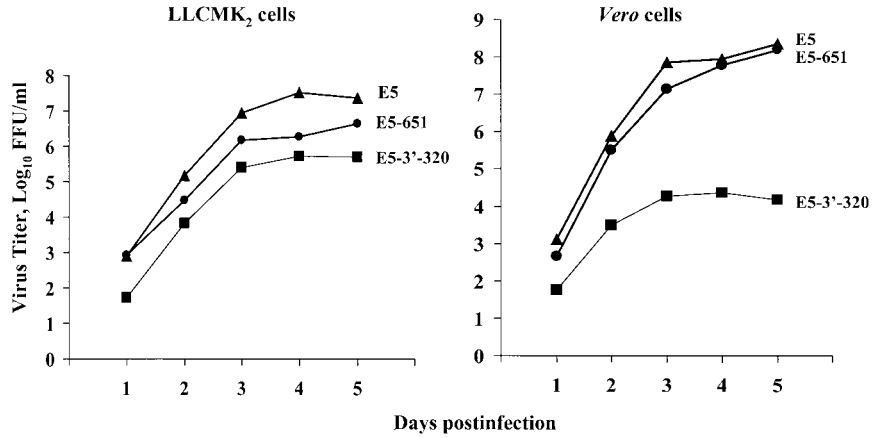

FIG. 2. Analysis of growth of parental E5 and its recombinant derivative viruses in simian $LLCMK_2$ and *Vero* cells. Cells were infected with the indicated virus at m.o.i. of 0.01 and following virus adsorption for 1 h, inoculum was removed and fresh medium was added. Virus in culture medium was harvested at indicated times and its titer was determined by a focus-forming assay on the respective cells as described under Materials and Methods.

Characterization of the rescued 3′-NCR deletion mutant

The rescued E5-3′-320 differed from the E5-651 clone with respect to plaque morphology in simian cells. The 3′-NCR deletion mutant failed to produce visible plaques on *Vero* cells, the cell substrate in which this virus was recovered (Table 2). The mutant produced plaques on $LLCMK_2$ cells, but they were very small, less than 0.2 mm. Such individual plaques were harvested and then subjected to three plaque-to-plaque passages in the $LLCMK_2$ cell line. Finally, seed stock suspension of E5-3′-320 virus was prepared by additional amplification in *Vero* cells. The complete genome sequence of the E5-3′-320 mutant was determined and compared with sequence of E5-651 clone. The only difference detected was the 3′-NCR deletion of the E5–3′-320 mutant.

The replication efficacy of parental E5, recombinant E5-651, and its 3′-NCR deletion mutant was assayed using $LLCMK_2$ or *Vero* cells inoculated with a multiplicity of infection (m.o.i.) of 0.01 (Fig. 2). In *Vero* cells growth of E5-3′-320 was 10^4 less than that of its E5-651 parent or E5 parent itself. The deletion mutant also grew less well in $LLCMK_2$ cells but it was restricted only 9- to 50-fold (Fig. 2 and Table 2).

Neuroinvasiveness of 3′-NCR deletion mutant in SCID mice

Neuroinvasiveness of E5-651 and its E5-3′-320 deletion mutant was evaluated in SCID mice in groups of five inoculated ip with decimal dilutions of virus. The recombinant 3′-NCR deletion mutant was less virulent for SCID mice than its immediate E5-651 parent. The estimated ip LD50 for E5-3′-320 was 479 PFU compared to 20.4 PFU for E5-651. Overall, the cloned E5-651 and E5-3′-320 viruses were 5100 and 119,750 times less neuroinvasive, respectively, than their wild-type progenitor, LGT TP21 virus.

Characterization of virus recovered from the brain of moribund SCID mice inoculated ip with E5 or its recombinant derivatives

Death of mice inoculated ip with E5-651 or E5-3′-320 was delayed by a factor of 2 compared to that of mice inoculated with their biologically derived E5 parent (Pletnev and Men, 1998; Campbell and Pletnev, 2000). This delay is consistent with the increase in attenuation of the recombinant viruses as measured by ip LD50 in SCID mice. In an attempt to explain the observed significant delay in onset of encephalitis, we sequenced the full genome of the brain isolates and compared the sequence to that of the virus that was inoculated ip (Table 3). The brain isolates differed from the virus used to initiate infection by at most three coding changes in E, NS1, NS2B, NS3, NS4B, or NS5. Three of the brain isolates sustained three coding changes, while the remaining two viruses differed from inoculated virus at only one amino acid position. Two brain isolates from SCID mice inoculated ip with 4.9 LD50 or 49 LD50 of recombinant E5-651 shared two coding changes, one at nt 1571 [G → A (Ala_{201} → Thr) or G → G/A (Ala_{201} → Ala/Thr)] and the other at nt 5794 [G → U (Glu_{399} → Asp) or G → G/U (Glu_{399} → Glu/Asp)]. All of the other seven coding changes were unique, i.e., not shared with other isolates. Clearly, additional study will be required to link any of these mutations to increased neuroinvasiveness and/or neurovirulence.

In an initial attempt to clarify this situation, a mouse brain isolate of E5-651 that contained the unique amino acid substitution (His_{130} → Tyr) in E (Table 3) was passaged once in *Vero* cells and then tested in five SCID mice by ip inoculation of 1 or 10 PFU. All inoculated mice died during the 14-day observation period. In addition,

TABLE 3

Non-Coding or Coding Changes in Virus Recovered from Brain of Moribund Mice 14 or 28 Days after ip Inoculation of E5 or Recombinant cDNA-Derived E5 (Clone E5-651 or Clone E5-3′-320)

		Deviation of sequence of brain isolate from indicated virus inoculated 14[a] or 28[b] days previously				
			Recombinant cDNA-derived clone E5-651			Recombinant cDNA-derived clone E5-3′-320, inoculum 2.1 LD_{50}[b]
Genome region	Nucleotide position	Uncloned E5, inoculum 16.7 LD_{50}[a]	Inoculum 4.9 LD_{50}[b]	Inoculum 49 LD_{50}[a]	Inoculum 490 LD_{50}[a]	
E	1071			C → C/U (Thr_{34} → Met)		
	1358				C → U (His_{130} → Tyr)	
	1416					G → A (Gly_{149} → Glu)
	1571		G → A (Ala_{201} → Thr)	G → G/A (Ala_{201} → Thr)		
	2175	U → U/C (Val_{402} → Ala)				
NS1	3381	U → U/C (Val_{308} → Ala)				
NS2A	3523		G → A	G → G/A		
	3952				A → U	
NS2B	4254		G → A (Ser_{17} → Asn)			
NS3	5518	C → C/U				
	5794		G → U (Glu_{399} → Asp)	G → G/U (Glu_{399} → Asp)		
	5896		A → G	A → A/G		
NS4B	7432		C → C/U			
	7455	C → C/U (Ala_{183} → Val)				
NS5	10333		C → U			

Note. Coding changes are positioned left of center whereas non-coding changes are positioned in the center of their respective columns.

the mouse brain isolate of E5-3′-320 virus that contained the unique amino acid substitution (Gly_{149} → Glu) in E (Table 3) and that was also passaged once in *Vero* cells was lethal for 100 or 80% of SCID mice inoculated ip with 1000 or 100 PFU, respectively. These findings suggest that the observed spontaneous mutations in neuroinvasive isolates of the recombinant E5-651 and E5-3′-320 are responsible for the increased neuroinvasiveness of these mutants in immunodeficient mice.

Immunogenicity and protective efficacy of parental E5 and its recombinant E5-651 or E5-3′-320 virus

Three-week-old outbred Swiss female mice (7–9 g) were inoculated ip with decimal dilutions of parental E5 or its cDNA-derived E5-651 or E5-3′-320 virus (Table 4). Twenty-two days after inoculation, immunized as well as nonimmunized (control) mice were bled to measure the titer of serum neutralizing antibodies against LGT TP21. All immunized mice seroconverted. Mice immunized ip with 10 PFU of parental virus or either of its recombinants developed a moderate to high titer of neutralizing antibodies against LGT TP21. However, immunization with 10- or 100-fold more virus increased the titer of TP21 neutralizing antibodies attained. There was no significant difference in the highest serum antibody titer induced by parental E5 or either of its recombinant derivatives in Swiss mice.

On day 23 postimmunization, mice were challenged ip with 2000 ip LD50 of the wild-type LGT TP21 strain. Mice immunized ip with 10 PFU of E5, or E5-651, or E5-3′-320 were completely protected against lethal TP21 challenge, whereas none of the control mice survived challenge. It is noteworthy that the more attenuated mutant (E5-3′-320) which exhibited restricted growth in cell culture and a significant reduction in neuroinvasiveness for SCID mice was able to induce complete protective immunity in immunocompetent mice at such a low immunization dose. These data provide a basis for proposing that the E5 3′ deletion mutant be considered a candidate vaccine strain that might serve as a stand-alone vaccine.

TABLE 4

Antibody Response and Protective Efficacy of LGT Virus Strains in Swiss Mice

Immunizing virus		Geometric mean of serum neutralizing antibody titer (reciprocal)[a]	Mortality after ip challenge with 2000 ip LD_{50} of TP21[b]
Strain	Dose (PFU)		
E5	10^3	448	0/5
	10^2	237	0/5
	10	110	0/10
E5-651	10^3	372	0/10
	10^2	152	0/10
	10	130	0/10
E5-3′-320	10^4	363	0/9
	10^3	282	0/10
	10^2	271	0/9
	10	225	0/10
Control	NA	<20	9/9

[a] Neutralizing antibodies in mouse serum collected 22 days after immunization were measured by a 50% focus reduction test using TP21 virus.

[b] Number of mice that died/number of mice tested.

Alternatively, this mutant could provide a foundation for further alteration to yield a live virus vaccine for use in preventing disease caused by antigenically related tick-borne flaviviruses.

DISCUSSION

Rescue of LGT strain E5 from full-length cDNA was achieved by replacing the coding region and short flanking conserved sequences of the TP21 genome with corresponding sequence of E5 strain in the stable infectious cDNA clone of wild-type LGT strain TP21 (Campbell and Pletnev, 2000). It should be noted that the seven amino acids of the consensus sequence that differentiate E5 from its more neurovirulent TP21 parent were retained in the rescued E5-651 clone. However, sequence analysis of the genome of the *Vero* cell culture-rescued E5-651 clone identified eight unique nucleotide differences from the consensus sequence of E5 virus that developed during the construction of infectious cDNA (Table 1). These changes were also present in the plasmid DNA from which the recovered clone was derived, providing evidence that the E5-651 clone was derived from cDNA. Only three of eight nucleotide differences resulted in an amino acid substitution in structural protein E or nonstructural protein NS4B. Virus rescued in certified *Vero* cells appeared to be somewhat more stable than virus rescued in primary chicken embryonic fibroblast cells. Following 12 passages of the cDNA-derived E5-651 clone in *Vero* cells, two new amino acid substitutions were identified, one in NS2B and the other in NS3. Additional variation in genome sequence of the CEF cell culture-recovered E5-651 clone also occurred after 12 passages in the CEF cell line (Table 1).

Similar to its E5 parent, the E5-651 clone was not neuroinvasive in normal adult mice when a large dose (10^6 PFU) was used for intraperitoneal inoculation. However, difference in neuroinvasiveness of E5-651 clone compared to its E5 parent became evident when the more permissive SCID mice were used for evaluation of this manifestation of virus virulence. The cloned E5-651 virus was 3.4×10^2-fold less virulent than its moderately attenuated E5 parent and 5.1×10^3-fold less neuroinvasive than its progenitor, strain TP21, the parent of E5 (Table 2). This further attenuation exhibited by the rescued E5-651 clone compared to its E5 parent was associated with a reduced cytopathic effect and plaque size in simian cell culture. One or more of the three unique amino acid substitutions in E and NS4B of the cloned E5-651 (Table 1) virus were responsible for reduced neuroinvasiveness of virus in SCID mice and for the restricted plaque phenotype in simian cells.

The 3′-noncoding region of tick-borne flaviviruses is approximately 400–800 nts in length and is predicted to form stem-and-loop secondary structures (Mandl *et al.,* 1991; Dobrikova and Pletnev, 1995; Wallner *et al.,* 1995; Mandl *et al.,* 1998) that are thought to play a crucial role in flavivirus replication (Men *et al.,* 1996; Blackwell and Brinton, 1997; Chen *et al.,* 1997; Zeng *et al.,* 1998). Recently, Mandl *et al.* (1998) provided evidence that certain deletions introduced into the 3′ NCR of TBEV genome reduced replicative capacity in cell culture and virulence for mice. The last 340 nts of the 3′-terminal sequence of TBEV strains (designed as "core element") are more conserved than the sequence located in the 5′ end of the 3′-NCR of the genome ("variable region") that starts just after the stop codon of the long open reading frame and ends just before the core element. The core element sequence of the European subtype (strain Neudoerfl) or Far Eastern subtype (strain Sofjin) of TBEV is 81 to 82% homologous to the terminal 340 nts of LGT. In addition, there is a 94.6–95.8% homology in sequence of the last 95 nts of LGT and the corresponding region of various TBEV strains. This region can potentially form a stem-and-loop RNA structure that is thought to be essential for viral replication. For this reason, we attempted to achieve greater attenuation of LGT E5 by engineering a 320, 374, 449, or 471 nt deletion that included the variable region and extended into the core element of the 3′NCR of clone E5-651 (Fig. 1). Only the cDNA construct with a 320 nt deletion between nts 10379 and 10700 yielded RNA transcripts that were infectious when transfected into certified *Vero* cells. The larger deletions were lethal. This indicated that the minimum 3′-NCR terminal sequence required for viability of LGT virus was more than 190 nucleotides (as in the nonviable construct pE5-3′-374; Fig. 1) but not more than 244 nucleotides (as in the viable construct pE5-3′-320). In contrast, the minimum requirement for DEN4 virus viability was the last 113 nts at the 3′ end (Men *et al.,* 1996), while for TBEV, viability required a minimum sequence 222 nts in length at the 3′ end of genome (Mandl *et al.,* 1998).

The genetic stability of the 3′-NCR sequence of the E5-3′-320 deletion mutant, its parental E5-651, and E5 virus itself as well as wild-type LGT TP21 strain and its cDNA-generated TP21-656 clone (Campbell and Pletnev, 2000) was monitored during long-term serial passage in *Vero*, $LLCMK_2$, or CEF cells. Sequence of the last 590 nts of these virus genomes was found to be completely conserved after cultivation in simian *Vero* or $LLCMK_2$ cells. It was also true for the 3′ end genomic sequence of E5, E5-651, and E5-3′-320 viruses passaged 12 times in avian CEF cells. In contrast, in CEF cells the genome of TP21 virus and, more surprisingly, the genome of cDNA-derived TP21-656 clone was spontaneously modified by the insertion of a poly(A) track (approximately 100–120 nt in length) into the variable region of the 3′ end. The presence of a poly(A) track in the TBEV genome had already been described as a spontaneous mutation in certain TBEV strains (Wallner *et al.,* 1995).

Analysis of plaque morphology and growth yield showed that the viable E5-3′-320 deletion mutant exhib-

ited more restriction in replication on either simian cell line than that its immediate E5-651 parent (Fig. 2, Table 2). The deletion mutant was noncytopathic in *Vero* cells and exhibited a reduced growth capacity of 4 orders of magnitude compared to the parental E5 or E5-651 clone. Growth restriction in cell culture has also observed for the 3′-NCR deletion mutants of DEN4 virus (Men *et al.,* 1996) and TBEV (R-Nd/3′Δ10919; Mandl *et al.,* 1998). This suggests that most or the entire 3′-NCR sequence is necessary for efficient virus replication.

Finally, neuroinvasiveness of the 3′-NCR deletion mutant was evaluated in the highly permissive SCID mouse model. Table 2 indicates that the reduction of replication of E5-3′-320 in simian cell culture compared to its immediate E5-651 parent was also associated with an additional 23.5-fold attenuation in immunodeficient mice. The E5-3′-320 mutant was 1.2×10^5 times more attenuated for SCID mice than its progenitor, TP21, and approximately 8×10^3 times less neuroinvasive in mice than E5 virus, which had been considered previously as a potential live virus vaccine candidate for protection against disease caused by tick-borne flaviviruses (Price *et al.,* 1970). It should be noted that the live E5 virus vaccine candidate exhibited low neurovirulence in monkeys; in fact it induced fewer CNS lesions than the 17D vaccine strain of yellow fever virus, which is considered to be one of the safest live human vaccine viruses (Nathanson *et al.,* 1968). Evaluation of the E5 strain in human volunteers (Price *et al.,* 1970) suggested its potential value for the protection against human illness caused by related tick-borne flaviviruses.

Ideally, a live attenuated flavivirus vaccine should be completely free of detectable neuroinvasiveness, but this is often difficult to achieve. For example, the licensed yellow fever 17D vaccine is able to invade the CNS of infant CD-1 mice from a peripheral site of inoculation and to cause death (Guirafhoo *et al.,* 1999). Vaccine-associated encephalitis and death of recipients following immunization with the 17D yellow fever vaccine accidentally occurred during early clinical trials (Monath and Heinz, 1996; Jennings *et al.,* 1994; Merlo *et al.,* 1993). In the present study the highly attenuated E5-3′-320 deletion mutant retained its ability to invade the CNS of immunodeficient mice and caused severe disease, possibly as a consequence of a spontaneous single amino acid substitution (a reversion $Gly_{149} \rightarrow Glu$) in the E glycoprotein (Table 3). Interestingly, the mutation at codon GGG (1415–1417 nts) to AGG in the sequence of the E5-651 virus leading to amino acid substitution Gly_{149} to Arg in the E protein was also detected when E5-651 virus was rescued from the full-length infectious cDNA clone in CEF cells and this became the predominant sequence following 12 passages in CEF cells (Table 1). Three of five SCID mice inoculated ip with 10 PFU of CEF cell culture-derived E5-651 (passage 1) died during the 7-week observed period, whereas deaths were not observed when 10 PFU of *Vero* cell culture-derived E5-651 virus (passage 1) was employed for ip inoculation of five SCID mice. This suggests that the CEF cell culture-derived virus in which Gly and Arg fluctuate at position 149 in E protein was more virulent than the *Vero* cell culture-derived virus, which contains only a Gly residue at position 149 in E protein. This observation considered together with the reversion of $Gly_{149} \rightarrow Glu$ observed in the brain-adapted E5-3′-320 virus suggests that amino acid substitution at position 149 in E protein might have an important effect on neuroinvasiveness. The amino acid substitution at position 149 is located in the loop E_0F_0 of central domain I in the three-dimensional structure of the E protein which has been proposed to play role in determination of flavivirus virulence (Rey *et al.,* 1995; McMinn, 1997). Also, the $Asn_{154} \rightarrow Leu$ mutation in the glycosylation site that is located in the loop E_0F_0 of E protein domain I significantly decreased neurovirulence of the TBEV/DEN4 chimeric virus in mice and restricted virus replication in cell culture (Pletnev *et al.,* 1993). In the present study, the amino acid changes that occurred in the same loop of domain I of E protein at position 149 in LGT E5 derivatives (a change $Glu_{149} \rightarrow Gly$ in the Vero cell culture-rescued E5-651 virus, a change $Gly_{149} \rightarrow Arg$ in the CEF cell culture-rescued E5-651 virus or a reverse mutation $Gly_{149} \rightarrow Glu$ in the mouse brain-adapted E5-3′-320 virus; Tables 1 and 3) also affected virus neuroinvasiveness for mice. These changes in E glycoprotein should be included in the menu of assays to be performed when vaccine strains are monitored for safety.

Our findings suggest that a high degree of LGT virus attenuation is possible to achieve by the gradual accumulation of the amino acid changes in a single virus following the pathway: TP21 → E5 → E5-651 → E5-3′-320, where the last step in genetic modification, i.e., the introduction of deletion into the 3′-NCR of genome, is the most crucial event in terms of reduced growth capacity of virus in cell culture and virus neuroinvasiveness.

The requisite balance between attenuation and immunogenicity of live virus candidate vaccines is often difficult to achieve. In the present study, the significant additional attenuation of both recombinant E5 viruses for immunodeficient mice was not accompanied by diminution of immunogenicity or protective efficacy. Complete protection of mice against challenge with wild-type LGT strain TP21 was observed when a very low dose of virus was used for immunization (10 PFU). Previous studies in mice (Price and Thind, 1973; Pletnev *et al.,* 2000) demonstrated a tight correlation between the level of neutralizing antibodies to LGT virus and cross-resistance to challenge with other members of the TBEV complex. Based on our data, it appears that the 3′-NCR deletion mutant of E5 has a favorable vaccine profile. However, more extensive evaluation of immunogenicity and protective efficacy of this vaccine candidate against disease caused by TBEV in mice and monkeys should be performed in the future.

MATERIALS AND METHODS

Cells and virus preparations

Certified *Vero* cells (WHO Seed, 143 passage) were obtained from Novavax Inc. (Rockville, MD). Primary chicken embryonic fibroblast (CEF) cells were kindly provided by Dr. Linda Wyatt (NIAID, NIH, Bethesda, MD). Simian $LLCMK_2$ cells were purchased from the American Type Culture Collection. Cells were grown in MEM with 1% glutamine, 10% fetal bovine serum, 50 μg/ml gentamicin, 0.25 μg/ml fungizone at 37°C and 5% CO_2. Virus stocks of the LGT wild-type strain TP21 and its further attenuated E5 mutants were prepared in *Vero* cells as described (Campbell and Pletnev, 2000).

Reverse transcription

Virus present in cell culture supernatant medium was precipitated with 8% polyethylene glycol 8000 (U.S. Biochemicals, Cleveland, OH) and 0.4 M NaCl overnight at 4°C and concentrated by centrifugation. Total RNA was extracted from virions using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). Reverse transcription (RT) was performed with SuperScript II Preamplification system (Life Technologies, Gaithersburg, MD) and an oligonucleotide (oligo) (1445) 5′-GCCTGCGGAG*GGTACCGATATC*AGCGGGTGTTTTTCCGAGACACG that is complementary to the LGT sequence at its 3′ terminus, i.e., nucleotides (nts) 10921–10943, and contains the *Eco*RV and *Kpn*I site immediately following the LGT 3′ end sequence. Prior to reverse transcription, 5–10 μg of RNA and 100 ng of primer were incubated at 70°C for 5 min and then chilled on ice. RT reaction mixtures contained this heat-denatured RNA plus ingredients of Superscript II kit and 200 U reverse transcriptase in a final volume of 100 μl. The reaction mixtures were incubated at 42°C for 2 h and then frozen and used as a template to generate double-stranded DNA by polymerase chain reaction (PCR).

PCR

The standard PCR mixture used to produce double-stranded cDNA contained 0.5 μM of each of the primer pairs, 200 ng plasmid DNA or 5–10 μl of RT product as a template, 400 μM of dNTPs, 1× buffer, and 5 U Takara LA *Taq* DNA polymerase (Takara LA PCR Kit, PanVera Co., Madison, WI) in a total reaction volume of 100 μl. The reaction mixture was preheated to 94°C for 2 min and then subjected to 30 cycles, each cycle being 98°C for 20 s and 68°C for 15 min.

Sequence analysis of viral genome

The complete sequence of the genome of (i) E5, (ii) E5 recovered from cDNA, and (iii) E5 or its derivatives isolated from the brain of moribund mice on day 14 or 28 postinfection was determined by sequence analysis of four overlapping RT-PCR cDNA fragments which were derived directly from virus RNA. The oligo 1445 was used as a primer to obtain the first-strand cDNA by reverse transcription as described above. PCR was performed to amplify the four overlapping genome fragments: A (nt 1 to 4192), B (nt 3491 to 7277), C (nt 6131 to 9669), and D (nt 8857 to 10943) using appropriate primers and Takara LA PCR kit. Primers for PCR and sequence analysis were designed using previously published LGT sequence (GenBank accession nos. AF253419 and AF253420). PCR products were purified in an agarose gel and isolated using a Qiagen gel extraction kit. Sequence of RT-PCR fragments was determined using BigDye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems/ABI Prism, Foster City, CA) and a Model 310 Genetic Analyzer.

Construction of full-length cDNA clones

1. Construction of full-length cDNA of LGT E5 (Fig. 1A). A long RT-PCR cDNA fragment of E5 genome and plasmid pTP21-636, which contains full-length infectious cDNA of LGT TP21, was used for this purpose (Campbell and Pletnev, 2000). Plasmid pTP21-636 was digested with *Sfi*I and *AgeI,* whose sites were unique in this vector. Almost full-length cDNA (~10.5 kb) of E5 genome was produced (Fig. 1A) by high-fidelity PCR using a positive-sense primer 1484 that contained the first 23 nts of LGT sequence, and a negative-sense primer 1107 that was complementary to the LGT nts 10439–10460 of the 3′ terminus. This RT-PCR fragment was derived from a E5 virus suspension of low titer (1.2×10^4 PFU/ml). The PCR product (LGT E5 nts 1–10460) was digested with *Sfi*I and *AgeI* and then cloned into the *Sfi*I(133)–*Age*I(9737) part of the pTP21-636 vector (nucleotide numbers indicate the first base of the recognition site sequence and correspond to the full-length sequence of LGT genome), which contained a *Not*I cleavage site, SP6 promoter, and the first 133 nts and nts 9737–10943 of LGT TP21 genome that are completely conserved in E5. There were also *Eco*RV and *Kpn*I cleavage sites, which were incorporated into the LGT cDNA immediately downstream of the 3′end of genome (Fig. 1). The resulting pE5 clones that contained LGT E5 were identified by restriction enzyme analysis and sequence analysis of genomic regions where TP21 and E5 genomes differed. Six individual full-length E5 cDNA clones (plasmids pE5) were stable in the plasmid vector when propagated in *Escherichia coli*. The complete viral sequence one of these six plasmids was determined; this plasmid was designated as pE5-651 and the corresponding number was used to designate rescued virus.

2. Introduction of deletions into full-length LGT E5 cDNA (Figs. 1B and 1C). To introduce deletions into the 3′ noncoding region of the E5 genome, PCR-generated subfragments between the *AgeI* site (nt 9737) and the

*Kpn*I site at the 3′ end were cloned in the p624-3 vector (Campbell and Pletnev, 2000), which contains these unique cleavage sites. The unique *Afl*II site (nt 10379) was introduced downstream of the TAA-stop codon in the 3′ NCR of E5-651 genome (Figs. 1B and 1C). The first PCR-amplified fragment was produced using pE5-651 plasmid as a template and the positive-sense primer (oligo 1013) that contains 9603–9623 nts of LGT genome and the negative-sense primer (oligo 1638) 5′-TTGGACTCCTTG**CTTAAG**GCTTTAAAATATTGAGCTCTC (the *Afl*II site sequence is in bold and the stop anticodon is underlined). This PCR fragment was digested with *Age*I and *Afl*II. Four deletions downstream of *Afl*II site, 320, 374, 449, or 471 nts in length were introduced by PCR using the negative-sense primer (oligo 1445), which contains the complementary sequence of the last 23 nts at the LGT 3′ terminus and the *Eco*RV and *Kpn*I site and the positive-sense primer (oligo 1640, 1641, 1642, or 1643), which contains the targeted deletion sequence and the flanking *Afl*II site. The sequence of these mutagenic primers were as follows: for the 320 nt deletion between 10379 and 10700 nts, oligo 1640, 5′-ACTGGGCGTTAT*CTTAAG*GCCCCAGGGGGGAAACCCCTG; for the 374 nt deletion between 10379 and 10754 nts, oligo 1641, 5′-GGATATTTCCTC*CTTAAG*ATACCAAATGTCCCCTCGTCA; for the 449 nt deletion between 10379 and 10829 nts, oligo 1642, 5′-CCCCTCGTCAGA*CTTAAG*GGGGGGCGGTTCTTGTTCTCC; for the 471 nt deletion between 10379 and 10851 nts, oligo 1643, 5′-ACGGACGTGCGC*CTTAAG*AAACTTTGTGAGACCCCTTGC. PCR fragments were digested with *Afl*II and *Kpn*I, and then cloned into the *Age*I–*Kpn*I-digested region of the p624-3 vector (Campbell and Pletnev, 2000) together with an *Age*I–*Afl*II-fragment derived from first PCR as described above. After transformation of bacteria with ligation mixtures, the clones (p624-3′-320, p624-3′-374, p624-3′-449, and p624-3′-471) that contained the targeted deletions in the 3′-NCR of genome were identified by sequence analysis. Construction of full-length E5 cDNA clones with deletions in 3′-NCR of genome was completed by ligation of plasmid p624-3′-320, p624-3′-374, p624-3′-449, or p624-3′-471, which had been digested with *Not*I and *AgeI*, and the *Not*I–*Age*I fragment of pE5-651. The stable individual full-length E5 cDNA clones (pE5-3′-320, pE5-3′-374, pE5-3′-449, and pE5-3′-471), which contained deletion of 320, 374, 449, or 471 nts in length at 3′-NCR of genome, were identified by sequence analysis.

RNA transcription, transfection, and recovery of virus

Each of the stable pE5 plasmids containing full-length LGT cDNA (Fig. 1) was linearized with *Eco*RV, extracted with phenol-chloroform, and ethanol precipitated. For *in vitro* RNA synthesis, the transcription reaction mixture contained 5 μg of linearized DNA; 1 mM cap analog $m^7G(5')ppp(5')G$ (New England BioLabs, Beverly, MA); 0.5 mM each ATP, CTP, and UTP; 10 mM DTT; 1× polymerase buffer; 100 U of RNase inhibitor; 50 U of SP6 RNA polymerase (Promega, Madison, WI) in a volume of 100 μl. The reaction mixture was incubated at 37°C for 1 h, and the DNA template was then digested with 3 U of RQ1 DNase (Promega) for 10 min at 37°C. The typical yield of RNA was approximately 10 μg as determined by agarose gel electrophoresis analysis.

RNA transcripts of the full-length LGT constructs were used to transfect subconfluent monolayers of CEF cells or simian *Vero* cells in the presence of transfection reagent LipofectAmine (GIBCO BRL, Gaithersburg, MD). On day 5 and again on days 10, 15, and 20, cells were split and passaged. Cells cultured in slide chambers were examined on each of these occasions by immunofluorescence assay (IFA) for the presence of LGT proteins using a LGT-specific mouse antiserum or LGT-specific hyperimmune mouse ascitic fluid. When 80–100% of cells were positive by IFA, the contents of infected T-75 flasks were collected, frozen, and later used for characterization of cDNA-derived LGT virus. These recombinant LGT viruses were amplified only once in simian *Vero* or CEF cells, after which viral RNA was isolated and reverse transcribed for cDNA amplification and sequence analysis. In a similar manner, the sequence of the *Vero* cell-derived or CEF cell-derived clone E5-651 was determined after an additional 11 passages in the corresponding cell line or after plaque-to-plaque purification on $LLCMK_2$ cells and one round of amplification on *Vero* cells.

The procedures used for plaque assay and analysis of replication in cell culture were described earlier (Pletnev *et al.,* 1992, 1993). Also, the immunostaining focus-forming assay (Ishimine *et al.,* 1987) was used in parallel with the plaque assay for determination of virus titer, because the recombinant viruses did not produce distinct plaques in *Vero* cells. Serial 10-fold dilution of virus suspension in MEM containing 2% heat-inactivated fetal bovine serum (FBS) were inoculated (0.2 ml) into duplicate wells of 6-well or 24-well tissue culture plates containing monolayer of *Vero* or $LLCMK_2$ cells. After 1 h of absorption at 37°C, inoculum was removed and cells in 6-well plates were overlaid with agar and stained to reveal plaques with neutral red 7 days later. Cells in 24-well plates were overlaid with MEM containing 2% FBS, 50 μg/ml gentamicin, 0.25 μg/ml fungizone, and 1% tragacanth gum (Sigma Chemical Co., St. Louis, MO) and incubated for 4 days at 37°C and 5% CO_2. Medium was removed, and the cell monolayers fixed for 30 min with methyl alcohol and rinsed twice with PBS. Cells in the wells were treated sequentially with 1:1000 diluted LGT-specific mouse antibodies and peroxidase-labeled polymer conjugated to anti-mouse immunoglobulins (Dako Co., Carpinteria, CA) diluted 1:10 in PBS. Antibody-bound foci of infectious cells were developed using 0.01% H_2O_2 and 0.04% 3,3′-diaminobenzidine (Sigma) in PBS and

counted, and virus titer was expressed as a focus-forming unit per milliliter (FFU/ml).

Evaluation of cDNA-derived viruses in mice

In a previous study it was observed that immunodeficient (SCID) mice were 10^7 to 10^8 times more sensitive for detection of neuroinvasiveness than outbred Swiss mice (Pletnev and Men, 1998). For this reason SCID mice were used for assay of neuroinvasiveness of LGT cDNA-derived virus E5 and its 3′-NCR deletion mutant. In this assay, female 3-week-old C.B.-17 Icr/*scid*/*scid* mice (Taconic Farms, Germantown, NY) in groups of 5 were inoculated ip with decimal dilutions of cDNA-derived E5 (clone 651) and its 3′-NCR deletion mutant. These mice were observed for mortality for 7 weeks.

Blood, liver, and brain of moribund mice which exhibited signs of advanced encephalitis were harvested and a 10% tissue suspension was prepared in MEM, frozen, and later used for virus isolation and sequence analysis. Titer of virus in these tissue suspensions was determined by plaque assay or focus-forming assay using monolayers of *Vero* or $LLCMK_2$ cells. One hundred microliters of the 10% tissue suspension was used for isolation of RNA, reverse transcription, cDNA amplification by PCR, and sequence analysis of recovered virus as described above. Also, the mouse brain isolate of E5-651 or E5-3′-320 virus was amplified in *Vero* cells and evaluated in the three-week-old SCID mice in groups of 5 that were inoculated ip with decimal dilutions of virus.

Immunogenicity of parental E5 and recombinant E5-651 or E5-3′-320 viruses was evaluated in three-week-old female Swiss mice that were inoculated ip with 10, 10^2, 10^3, or 10^4 PFU. On day 22 postinoculation, mice were bled to evaluate antibody response, challenged ip the next day with 2000 ip LD50 of TP21 virus, and observed for an additional 4 weeks.

For determination of LGT virus-neutralizing antibody titers, 10-fold diluted serum was heat inactivated for 30 min at 56°C. Serial twofold dilutions of serum (starting at a serum dilution of 1:10) were mixed with equal volume of TP21 virus suspension containing approximately 100 FFU. The mixture was incubated for 30 min at 37°C, and 0.1 ml was added to duplicate wells of $LLCMK_2$ cells in a 24-well plate. After 1 h of absorption at 37°C, inoculum was removed and cells were overlaid with MEM containing 2% FBS, 50 μg/ml gentamicin, 0.25 μg/ml fungizone, and 1% tragacanth gum and titrated for infectious virus using the focus-forming assay as described above. Antibody titer was the highest dilution of antibody that reduced focus formation 50% compared to serum collected from nonimmunized mouse.

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